Master thesis of Tugce Arslan Özdemir

POLLEN AND SEED LONGEVITY IN GM AND NON-GM WHEAT

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1 Abstract

The objective of this study was to investigate some life history traits, namely, seed and pollen longevity, and outcrossing ability of spring wheat lines genetically modified (GM) for the resistance to powdery mildew compared to conventional (non-GM) wheat lines and varieties. For this purpose three experiments were carried out in the glasshouse. In the first experiment the seeds of six transgenic and nine conventional wheat lines and varieties were used in order to assess the germination ability of the seeds before and after storage for three and six months in different humidity and oxygen conditions. In the second experiment, pollen viability and longevity of three transgenic and three control wheat lines and varieties was studied under different light and temperature storage conditions. In the last experiment, crosses were made between six transgenic and conventional wheat lines and varieties in order to assess their outcrossing ability and to obtain hybrids.

The results overall indicated that transgenic wheat lines do not differ from corresponding control lines in seed and pollen viability and longevity at the conditions of storage used in the experiments. Wheat pollen was viable after 9 hours of storage at 12% relative humidity. The number of hybrid seeds obtained was lower when the transgenic *Pm3b*#2 line was used as a pollen source. Since the quality of pollen was good, this might indicate lower pollen quantity produced by this line.

2 Introduction

Along with a growing number of GM crops proposed for farming in some countries, there is a public and ecological concern that transgenic plants may pose risks for the environment e.g. when escaping from the field where they were initially grown, becoming agricultural weeds and invasive plants in natural habitats or outcrossing with conventionally bred varieties and contaminating them with a transgene (Williamson, Perrins & Fitter 1990; Waines & Hegde 2003; Matus-Cádiz *et al.* 2004; Hanson *et al.* 2005; Andow & Zwahlen 2006; Matus-Cádiz, Hucl & Dupuis 2007).

Genetic engineering opens the door to new strategies for managing fungal diseases. Scientists are currently developing biotechnological techniques to conferring resistance to fusarium, mildew and other pathogens and are testing their effectiveness on different agricultural crops (Bliffeld *et al.* 1999; Bieri, Potrykus & Fütterer 2003; Srichumpa *et al*. 2005; Jones 2005).

In Switzerland transgenic lines of spring wheat (*Triticum aestivum*) were generated in laboratories of the ETH (Bieri, Potrykus & Futterer 2003) and the University of Zurich (Srichumpa *et al*. 2005). These lines were genetically engineered to carry the genes of resistance to powdery mildew (*Blumeria graminis* f.sp. tritici). Powdery mildew is a fungal disease that often causes problems for wheat growers and can spread rapidly, especially under mild and moist weather conditions (McMullen & Lamey 2001). In 2008 the first big field trial, investigating mildew resistance, ecological performance and possible environmental risks of these transgenic lines, started in Zurich-Reckenholz.

Wheat has no close relatives in natural habitats in Switzerland, therefore the main concerns are the gene flow between transgenic and conventional varieties, which may occur in the field, and seed bank in the soil after harvest which can lead to transgenic wheat volunteering in the field fallows next year and possible becoming a new agricultural weed if the transgene gives any fitness advantage to the plants (Williamson, Perrins & Fitter 1990; Conner, Glare & Nap 2003; Wilson, Dahl 2005; Kulikov, M., 2005; Andow & Zwahlen 2006). A transgene introduced into the plant genome can influence other, non-targeted traits, increasing or decreasing plant fitness (Wolfenbarger & Phifer 2000; Pilson & Prendeville 2004) and possibly changing life history traits.

In this context, an important issue is to investigate pollen and seed longevity, and outcrossing ability of newly obtained transgenic lines. Since pollen and seed survival and germination ability may highly depend on the environmental factors, such as moisture or temperature, this should be studied across different storage conditions and time intervals.
Wheat is used in agriculture for ages, therefore the biology of this crop is well studied.
Wheat is an annual or biennial grass having erect flower spikes and light brown grains sown in spring or in autumn. It flowers from the end of May to the beginning of June. This crop is grown in the areas where annual temperatures of 4.9 to 27.8°C prevail (Patnaik & Khurana 2003; Snow *et al.* 2005; Australian Government).

Duration of growth stages of wheat		
Plant growth stage	**Temperature requirements (C)**	**Duration (days)**
Germination	3-4 (minimum); 12–25 (optimum)	4–10
Flowering	14 (minimum)	
Vegetative : winter		280–350
Vegetative : spring		120–145

(Australian Government)

The flowering time of a wheat cultivar can last more than a week. It is known, that germination percentage of wheat seeds decrease gradually with an increase of the storage time (Malaker *et al.* 2008). The frequencies of dividing cells in wheat seeds were found to decrease with aging (Akhter *et al.* 1992). Minimum moisture for germination in wheat is 35–45% of kernel dry weight (Evans *et al.* 1975). The greatest differences in seed germination tended to be between 15 ˚C and 20 ˚C.

Seed viability decreases with increasing age, storage conditions. In particular, temperature and moisture have been suggested as the main factors influencing seed longevity. High temperature and moisture accelerate loss of seed viability in most species (Akhter *et al*. 1992; Ali *et al.* 2006).

It is believed that wheat pollen is viable only for 3 hours (De Vries 1971). Potential longevity of pollen increases with increasing temperature (Luna *et al.* 2001). According to the literature (Loureiro *et al*. 2007), wheat pollen can travel only 4–6 m from the source but sometimes these distances can be greater. Wind direction also affects pollen dispersal. It

is known, that humid weather makes pollen heavy and reduces the dispersal distances (Loureiro *et al*. 2007).

The duration of pollen viability is influenced by temperature and relative humidity: extremely cold or hot temperatures are unfavorable for pollination and fertilization (Waines, Hegde & 2003). Cool temperatures reduce the duration of pollen shed and pollen viability. However, for artificially stored pollen grains, cool temperatures and relatively high humidity levels prolong viability (Waines & Hegde 2003). Temperature has the greatest effect on pollen viability. In general, high temperatures reduce pollen viability (Welsh & Klatt 1971).

There is a lot of information in the literature about the biology of wheat. But seed and pollen longevity in the context of genetically modified wheat which has resistance against powdery mildew have never studied before.

We compared the lines of wheat genetically modified to be resistant to powdery mildew with corresponding conventional lines and varieties. We tested the seed and pollen longevity and the outcrossing ability of the wheat lines mentioned in the “Plant material” section below over different time intervals and storage and temperature conditions.

3 Material and Methods

3.1 Plant material

In our experiments we used the seeds of 15 lines and varieties of spring wheat:
1) Bobwhite SH 98 26 — conventional variety of spring wheat from Mexico, highly susceptible to powdery mildew;
2–5) Four Bobwhite transgenic lines *Pm3b*#1, *Pm3b*#2, *Pm3b*#3 and *Pm3b*#4 which have strain-specific resistance against powdery mildew, transgene *Pm3b* alleles were obtained from hexaploid wheat variety Chul (Yahiaoui *et al.* 2005);
6–9) Four sister lines S3b#1, S3b#2, S3b#3 and S3b#4 — isogenic lines (null-segregants), used as control lines for the *Pm3b*#1–4 GM lines mentioned above;
10) Frisal — old Swiss variety of spring wheat from which A9 *Chi* and A13 *Chi/Glu* transgenic lines were obtained;
11) A9 *Chi* transgenic line which carries chitinase gene from barley conferring non-specific quantitative resistance against fungal pathogens;

12) A13 *Chi/Glu* which carries chitinase and glucanase transgenes from barley conferring non-specific quantitative resistance against fungal pathogens;
13–15) three conventional Swiss wheat varieties Fiorina, Casana and Toronit commonly used in agriculture.

Transgenic lines *Pm3b*#1–4 were produced by biolistic transformation of Bobwhite SH 98 26 (Pellegrineschi *et al.* 2002; Zeller *et al*. 2010). The four *Pm3b* lines were derived from different transformation events and each of them carried a single copy of the transgene *Pm3b*.

Plants of the variety Frisal were transformed using the expression unit of plasmid MAGUCM (Bliffeld *et al.*1999) containing (for A9 *Chi/Glu* line) rice actin1 promoter (McElroy *et al.* 1990) barley seed β-1,3-glucanase coding sequence (HvGLU) (Leah *et al.* 1991) :: CaMV terminator and (for A9 *Chi* and A13 *Chi/Glu* lines) maize ubiquitin1 promoter (Christensen 1992) barley seed chitinase coding sequence (HvCHI) (Leah *et al.* 1991) :: CaMV terminator. One transgenic line was positively selected for *Chi* (line A9 *Chi*) and one for *Chi/Glu* (line A13 *Chi/Glu*) gene expression.

The seeds for the experiments were obtained from the wheat cluster sub-unit of the National Research Programme NFP59 (research groups of B. Keller, C. Sautter and B. Schmid).

3.2 Experimental design and methods

We performed three glasshouse experiments in order to test: (1) seed longevity of GM and non-GM wheat lines; (2) pollen longevity of GM and non-GM wheat lines; (3) outcrossing ability of GM and non-GM wheat lines.

3.2.1 Experiment 1: Seed longevity of GM and non-GM wheat lines.

In this experiment the following 15 wheat lines were used: Bobwhite, *Pm3b*#1, *S3b*#1, *Pm3b*#2, *S3b*#2, *Pm3b*#3, *S3b*#3, *Pm3b*#4, *S3b*#4, Frisal, A9 *Chi*, A13 *Chi/Glu*, Toronit, Fiorina, and Casana. The seeds for this experiment were obtained from the NFP59 field trial 2008 (Zeller *et al.* 2010).

We tested the longevity of the seeds from different transgenic and conventional wheat lines and varieties and their ability to germinate after storage under different environmental conditions.

Initial germination test

Initial germination tests were done in order to assess the germination rate of the seeds before storage. For the germination test five replicates were used. For one replicate 20 seeds were put into a Petri dish (Ø 10 cm) on filter paper sprayed with water (Fig. 1). The Petri dishes were randomized and sprayed with water regularly (every 2 days). Germination rate was recorded every third day, and the percentage of seeds germinated was calculated. Initial germination test took place in the glasshouse.

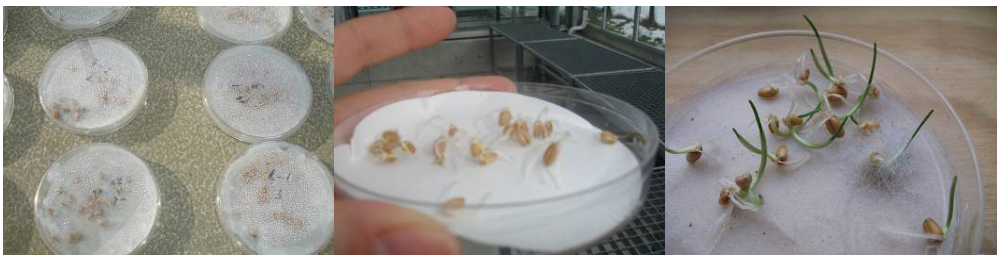


Fig.1. Initial germination test in Petri dishes.

Treatments maintained in the seed longevity experiment

Seed storage conditions (soil humidity and oxygen):

1) Wet soil aerobic
2) Dry soil aerobic
3) Wet soil anaerobic
4) Dry soil anaerobic

We provided four different soil storage conditions in order to find out how the longevity of the seeds changes after being stored in wet and dry soil, in the presence and absence of oxygen.

All the seeds were stored in the cold room (T = 6 ^{0}C) at the Institute of Evolutionary Biology and Environmental Studies, University of Zurich.

Time of storage:

1) Three months

2) Six months

We stored the seeds for 3 and 6 months in order to test how the germination ability of the seeds changes with time.

Nutrients obtained by mother plant:

1) No fertilizer (F1)
2) Fertilized twice during the growing season (F2)

Half of the plants (mother plants), from which the seeds for this experiment were obtained, did not receive fertilizer in the field (field trial NFP59 2008) and the other half had been fertilized twice during the year (Zeller *et al*. 2010). To the latter ones, fertilizer (3 g N m^{-2} as "Ammonsalpeter 27.5", Lonza, Visp, Switzerland) was applied twice during thr growing season. We incorporated these treatments of the mother plants into our experimental design and following analysis.

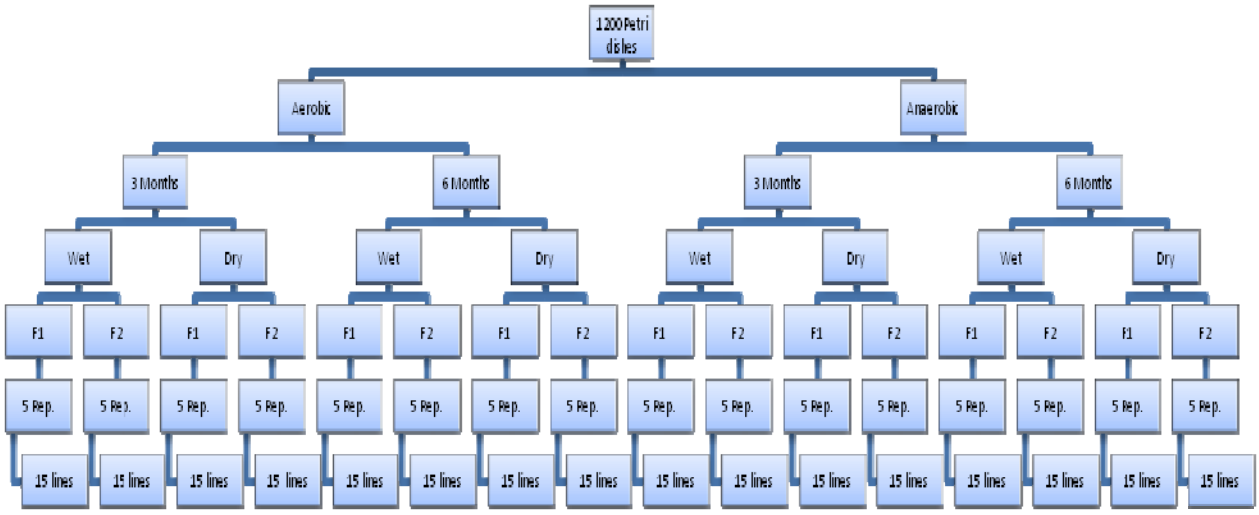


Fig.2. Design of the seed longevity experiment.

Half of the seeds were kept in dry soil which had 17.6% humidity and another half was stored in wet soil which had 77.5% humidity. We used the lawn soil from Ökohum. In order to maintain wet soil conditions (77.5% humidity), we added 200 ml of water per 1 l of soil. For the dry soil conditions nothing was added. The exact humidity was measured with a moisture sensor (Fig. 3).

Fig.3. Soil moisture sensor used in the experiment.

The soil was put into Petri dishes (Ø 10 cm). After this, the seeds were distributed on the soil surface and deepened into the soil so that they could not be seen (about 0.6 cm depth). The Petri dishes were closed with a tape (Fig. 4) and put into polypropylene bags (BIO HAZARD bags, Greiner), which had the size 600×780 mm and the wall thickness 0.05 mm.

Fig.4. The seeds in Petri dishes.

We maintained two different oxygen conditions: aerobic and anaerobic. In order to maintain the anaerobic conditions we replaced the air by nitrogen by evacuating the polypropylene bags with a water jet vacuum needle several times and filling the evacuated bag with nitrogen. The sulfur-free oxygen absorbers (ATCO FTM 2000S, Long life for art) and oxygen indicators (ageless-eye, Long life for art) were used in order to maintain the anaerobic conditions and ensure the stability of these conditions over the period of storage (Fig. 5). These oxygen absorbers are able to absorb 2000 ml oxygen (the oxygen from 10 l air) and release about 0.8 g of water during use.

Fig.5. Oxygen absorbers and anaerobic conditions maintained in a polypropylene bag.

One half of the Petri dishes was stored for 3 and another half − for 6 months. After this time, the germination test was done in order to assess seed survival rate after storage.

There were five replicates per mother plant nutrient level × variety × time of storage × soil storage conditions. Altogether 1200 Petri dishes were used (240 Petri dishes × five replicates).

20 seeds were placed into each Petri dish that resulted in 100 seeds per mother plant nutrient level × variety × time of storage × soil storage conditions.

30 Petri dishes together with two oxygen absorbers and one indicator were put into each polypropylene bag. Then all the bags were put into the boxes and stored in the cold room at 6 ^{O}C for 3 and 6 months. It was in the beginning of March 2009 when the boxes were placed in the cold room.

After 3 and six 6 months the seeds were taken out from the cold room and washed out from soil. The number of seeds germinated during the storage was recorded and expressed as percentage for each wheat line.

Second Germination Test

The second germination test was done to see if the viability of seeds changed after the storage and if the different environmental conditions affected seed survival. The seeds which did not germinate during storage were placed into Petri dishes. The germination test after storage was performed in the same way as the initial germination test. The seeds which did not germinate in the test were considered as those that lost their viability during

the storage. The second germination test took place in the plant room of the Institute of Evolutionary Biology and Environmental Studies (Fig. 6).

Fig.6. Second germination test in the plant room.

It was at the end of May 2009 when the 3 months old seeds were taken out and at the end of August 2009 when the 6 months old seeds was taken out from the cold room.

3.2.2 Experiment 2: Pollen longevity of GM and non-GM wheat lines

In this experiment the longevity of pollen of different GM and non-GM wheat lines was tested. The experiment took place in the glasshouse of the Institute of Evolutionary Biology and Environmental Studies. The seeds of six wheat lines and varieties were used in this experiment: *Pm3b*#1, *S3b*#1, *Pm3b*#2, *S3b*#2, Frisal and A9 *Chi*. The seeds were without contamination from other lines. The mother plants received fertilizer twice in the field (Zeller *et al.* 2010).

In the glasshouse, we grew the plants individually in 1-L black plastic pots from April 2009 until June 2009. The soil (same as for experiment 1) used in the experiment was mixed with fertilizer (by Osmocote), which is a coated NPK fertilizer that releases nitrogen, phosphate and potassium and trace elements over a pre-chosen period of time in the amount of 2 g per liter or soil. Soil and Osmocote were mixed in a mixing machine (Fig. 7).

Fig.7. The machine used for mixing soil and fertilizer.

There were five repetitions sown with 1-week time intervals between them. The pots of one repetition were put together as a block in the glasshouse compartment resulting in five blocks (blocks in time). The pots were labeled with the number of the wheat line, block and replicate number. The pots were randomized within blocks (Fig. 8). The border crop was sown as one additional row around each block to prevent edge effects. Wheat variety Fiorina was used for this purpose.

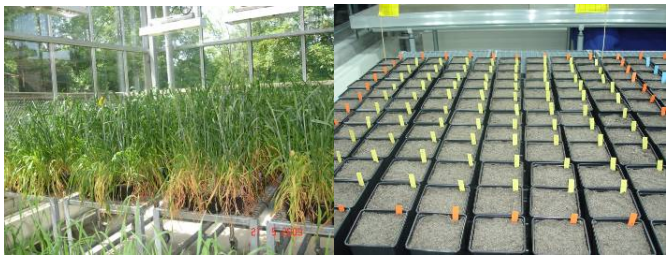


Fig.8. Pollen longevity experiment arrangement in the glasshouse.

After sowing the seeds, the pots were watered by hand from the top until they were wet and from then onwards they were watered daily. We put the seeds on the soil in the beginning of April 2009. The first plants of Frisal variety and A9 *Chi* line started flowering in the middle of May 2009. Bobwhite lines started flowering about 2 weeks later than Frisal. We started with pollen collecting and staining in June 2009 and continued for one month.

Treatments

Pollen storage conditions:

1) 10°C light
2) 10°C shade
3) 25°C light
4) 25°C shade

To see how the light and temperature affect pollen viability, pollen was stored at two different temperatures (10°C and 25°C) under different light conditions (light and shade). Shade was made using a plastic shading net which with small holes in it (Fig. 9).

All the treatments were applied in the climate chambers (Sanyo Versatile Environmental Test Chamber, model MLR); each time the humidity conditions were the same (45% humidity). There were 15 fluorescent lamps in the climate chambers each with 40 W.

Time of storage:

1) Immediately used for staining (no storage)
2) 20 min
3) 1 hour
4) 4 hours
5) 9 hours

Pollen was stored for different time periods before the staining was applied. The aim was to test how the pollen viability changes with time.

For each wheat line, we collected pollen from 10–15 flowering spikes (when more than half of the spike was flowering) from one experimental block at a time. The pollen was collected in a paper bag. Then this pollen was separated with tweezers into 17 parts and distributed into 17 Eppendorf tubes, 1.5 ml each. When we could see the pollen mass at the bottom of the tube it meant having enough pollen for staining. One tube with pollen was used for staining immediately; the other 16 tubes were put into plastic-tube trays and stored open in the climate chambers for a certain time at different temperature and light conditions. For maintaining shade conditions, the tray was covered from all sides with black plastic folio fixed on the tray by tape (Fig. 9).

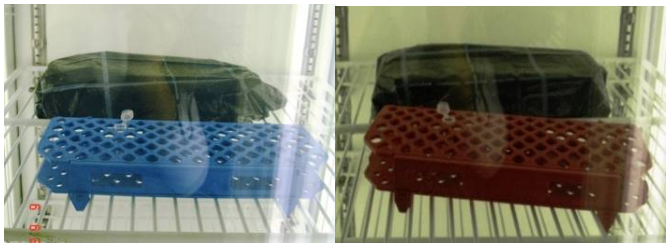


Fig.9. Treatments maintained in the climate chambers.

After storage for 20 minutes, 1 hour, 4 hours and 9 hours, respectively, one of the 16 tubes was removed from the tray and pollen was stained. In order to check pollen viability a pollen staining method was used. For this method (thiazolyl blue) MTT stain (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) was prepared. The test detects the presence of dehydrogenase. The test solution consisted of a 1% concentration of MTT in 5% sucrose (Rodriguez-Riano & Dafni 2000; Khotum & Flowers 1995; Norton 1966). MTT is reported as one of the best methods since it does not normally stain either killed or aborted pollen (Rodriguez-Riano & Dafni 2000). The MTT and sucrose were added to the distilled water and mixed on a 37°C heater. The solution was stored in the fridge during the experiment. The pollen grains were considered viable if they turned deep-pink or if they presented no colour but showed irregular black lines over their surface (Rodriguez-Riano & Dafni 2000).

Two Eppendorf tubes were taken from the two climate chambers (cold and warm) at a time. 1 ml of the MTT-sucrose solution was taken to an Eppendorf tube from the bottle (see Fig. 10a).

a) b)

Fig.10. a) MTT solution and **b)** solution and pollen on glass slides ready to be observed under light microscope.

The tube with stain solution was put onto a heater and heated up to 37 ^{0}C. Then a drop from this heated solution was put on a microscopic glass slide. The pollen was taken out from the tube and added into this drop. Cover slips were gently placed onto different slides for each wheat line. We waited for 10 minutes for pollen to be stained (Fig. 10b). The pollen on the slides was then observed under a phase contrast binocular microscope (Zeiss L-89) on the dark field (Fig. 11).

a) b)

Fig.11. a) Light microscope and **b)** observations of stained pollen.

The pollen grains which were stained deep-pink were counted as alive and the pollen grains which stayed yellow or white were counted as dead. There were also burst pollen grains which were not accounted for in the calculations for the analysis. Pollen was taken from 10−15 different plants of one variety from one block at each time (five blocks = five replicates). Since the blocks were planted with one week intervals, every day one variety from one block was tested. In total, the counting took six days for all the lines/varieties from one block. The pollen grains under the microscope were counted in two−three microscope views. In the end, in each microscope view about 100−200 pollen grains were counted.

3.2.3. Experiment 3: Outcrossing ability of GM and non-GM wheat lines

In this experiment the outcrossing ability of GM and non-GM wheat was tested in order to assess the productivity of different crosses between transgenic and conventional lines.

We used the seeds of the following lines: *Pm3b*#1, *S3b*#1, *Pm3b*#2, *S3b*#2, Frisal and A9 *Chi*. The seeds for the experiment were without contamination from other lines. 1-L pots were filled with soil which was used also in experiments 1 and 2. The soil was mixed with fertilizer (2 g of fertilizer [Osmocote, the same used in experiment 2] per liter of soil) in a mixing machine.

We grew the plants individually in 1-L black plastic pots in the glasshouse (see previous section for the glasshouse conditions) from the beginning of March until August 2009 when the plants became completely dry and the spikes were ripe. The seeds were sown 2−3 cm deep and the pots were watered until all soil was wet, later they were

watered every day. There were 10 replicates for each line. Insect traps were put to prevent insects spreading and harming the plants.

In the glasshouse, the pots with the lines from Bobwhite and Frisal varieties were arranged separately. The pots were completely randomized (within Bobwhite and Frisal) at the beginning but ordered later, when the plants started flowering, to make the crossings easier. After all the crossings were completed the pots were randomized again. Fiorina was used as a border crop to prevent edge effect.

Crosses were done between the lines under two complete mechanistic diallel schemes including reciprocal combinations (Tables 1−2). Artificial cross-pollination with pollen of the same wheat line (but taken from another plant) was done in order to have control for the effect of the crossing procedure itself. The crosses were made between S3b lines and *Pm3b* lines, and also between the Frisal mother variety and A9 *Chi*. We did not make any crosses between Bobwhite and Frisal.

♀ \ ♂	S3b#1	S3b#2	*Pm3b*#1	*Pm3b*#2
S3b#1	×	×	×	×
S3b#2	×	×	×	×
Pm3b#1	×	×	×	×
Pm3b#2	×	×	×	×

Table 1. The crossings between Bobwhite GM and non-GM lines.

♀ \ ♂	Frisal	A9 *Chi/Glu*
Frisal	×	×
A9 *Chi/Glu*	×	×

Table 2. The crossings between Frisal GM and non-GM lines.

We made 10 crosses per hybrid combination from the diallel tables. Then the pots were labeled with the number of the line, mother or father, and replicate number. Yellow labels represented mother plants, blue labels represented father plants for all varieties and the orange ones were for the border crop (Fig. 12).

Fig.12. Arrangement of Frisal varieties in the outcrossing experiment.

For the wheat plants which were used as mother plants, we waited until spikes were mature enough: the time when the spikes fully emerged but no anthers were visible outside the spikelets (Zadoks stage 55). The plants at Zadoks stage 65 (flowering of more than a half of the spike) were used as father plants (pollen source). The mother plant castration had to be done carefully and no anthers were left in the mother plant spike in order to prevent self-pollination. The tops of the cover leaves of the wheat flowers were carefully cut with scissors to make castration easier. All three of the premature anthers were gently (not to damage a stigma) removed from each flower of the spike with tweezers (Fig. 14).

Fig.14. The removal of the anthers (before and after the castration).

First, we did the crossings immediately after the castration of the mother plants but then we found out that better results were obtained when we waited for stigmas to be matured enough (for two days) before cross-pollination. Therefore, in the end, cross-pollination “without waiting” was done for the crosses of Frisal lines, which flowered earlier, and for ¼ of all the crosses of Bobwhite lines.

The castrated spike was covered with a paper bag in order to prevent pollination from other plants. For crossings with waiting, the plant was left for two days until the stigmas became matured and opened (Fig. 15). After this time, the top of the paper bag was cut and three spikes from father plant that had fresh pollen were put inside. For each crossing event we used 3 spikes from three different father plants (from the same line), then the spikes were shook and left in the bag to let them pollinate the stigmas. The bag was closed again together with three father spikes and fixed with staples. For crossings without waiting, the same was done immediately after mother spike castration.

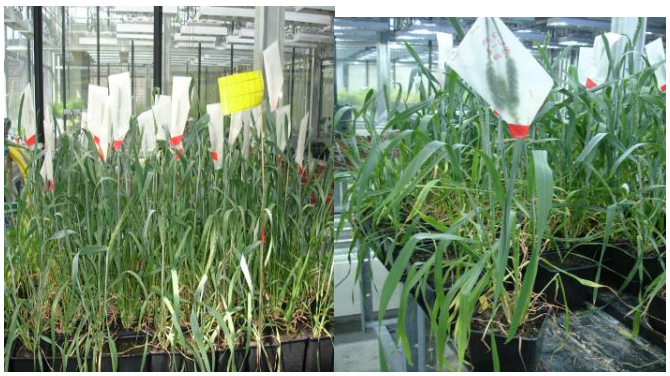


Fig.15. Wheat plants which were covered with a paper bag.

All of the plants, where the outcrossing was completed, were watered for two more weeks (every second or third day) and then left to dry out. After one month, all the plants had fully dried out and were harvested (Fig. 16). The number of successful crosses and the number of seeds produced were counted.

Fig.16. Harvested plants from outcrossing experiment and seeds obtained.

3.3 Analyses

Statistical analysis of the data was done with the software GenStat (VSN International Ltd.). GM and non-GM lines, different genotypes within these groups, Swiss and other wheat varieties were compared, all main effects of the treatments and all two-way interactions with treatments were added to the models.

3.3.1 Experiment 1: Seed longevity of GM and non-GM wheat lines.

In the seed longevity experiment we analyzed the percentage of seeds germinated out of all the seeds (response variable). First we analyzed initial germination ability of the seeds. Then we analyzed germination ability during storage. Figure 17 shows an example of the hierarchical model of all the comparisons made between different wheat lines and varieties. Effects of each treatment, two-way interactions of the treatments, effects of the lines and interactions of the lines and treatments were analyzed (Table 3).

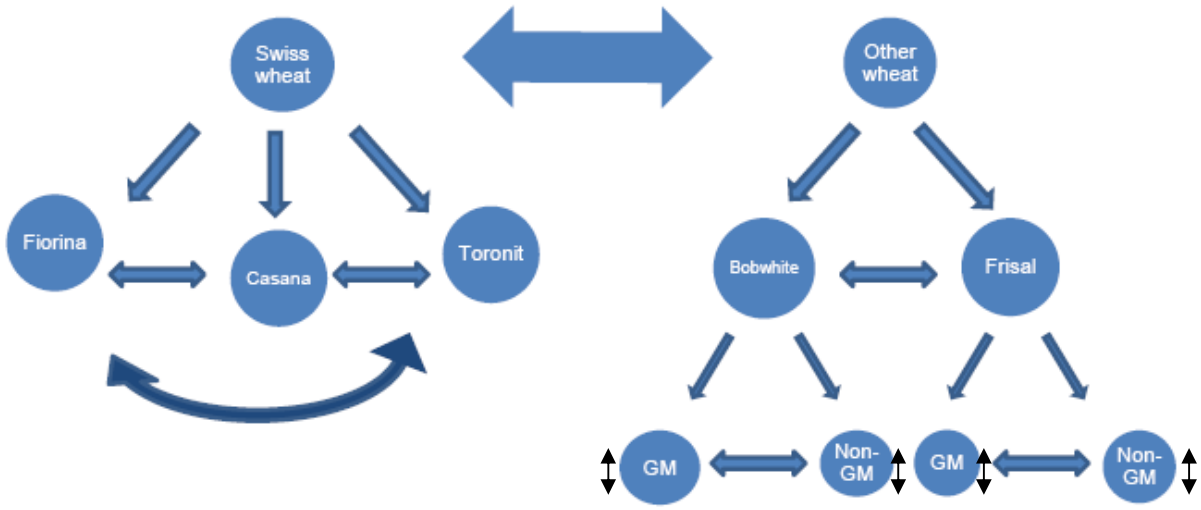


Fig.17. Hierarchical model of line comparisons of seed longevity experiment. Double-sided arrows show the comparisons with each other. Vertical arrows show the comparisons within GM and non-GM groups.

Table 3. Skeleton ANOVA table for the seed longevity experiment analysis

Source of variation	**d.f.**
Replicates	4

Treatments	
Time	1
Oxygen	1
Humidity	1
Fertilizer	1
Interactions of treatments	
Treatment Time × Oxygen	1
Treatment Time × Humidity	1
Treatment Oxygen × Humidity	1
Treatment Time × Fertilizer	1
Treatment Oxygen × Fertilizer	1
Treatment Humidity × Fertilizer	1
Line Contrasts	
Swiss vs other varieties	1
Within 3 Swiss varieties	2
Bobwhite vs Frisal	1
Within 2 Bobwhite control lines	1
Within 2 Bobwhite GM lines	1
Within 4 *Sb* lines	3
Within 4 *Pm3b* lines	3
Frisal control vs. GM	1
A9 *Chi* vs. A13 *Chi*/*Glu*	1
Interactions Line × Treatments	
Time × Swiss varieties vs. Other varieties	1
Time × Within Swiss varieties	2
Time × Bobwhite vs. Frisal	1
Time × Bobwhite mother variety vs. all the lines produced out of it	1
Time × Bobwhite control vs. GM	1
Time × Within Bobwhite control lines	3
Time × Within Bobwhite GM lines	3
Time × Frisal control vs. GM	1
Time × A9 vs. A13 (Within Frisal GM lines)	1
Oxygen × Swiss varieties vs. Other varieties	1
Oxygen × Within Swiss varieties	2
Oxygen × Bobwhite vs. Frisal	1
Oxygen × Bobwhite mother variety vs. all the lines produced out of it	1
Oxygen × Bobwhite control vs. GM	1
Oxygen × Within Bobwhite control lines	3

Continuation of Table 3

Change	d.f.
Oxygen × Within Bobwhite GM lines	3
Oxygen × Frisal control vs. GM	1
Oxygen × A9 vs. A13 (Within Frisal GM lines)	1
Humidity × Swiss varieties vs. Other varieties	1
Humidity × Within Swiss varieties	2
Humidity × Bobwhite vs. Frisal	1
Humidity × Bobwhite mother variety vs. all the lines produced out of it	1
Humidity × Bobwhite control vs. GM	1
Humidity × Within Bobwhite control lines	3
Humidity × Within Bobwhite GM lines	3
Humidity × Frisal control vs. GM	1
Humidity × A9 vs A13 (Within Frisal GM lines)	1
Fertilizer × Swiss varieties vs. Other varieties	1
Fertilizer × Within Swiss varieties	2
Fertilizer × Bobwhite vs. Frisal	1
Fertilizer × Bobwhite mother variety vs. all the lines produced out of it	1
Fertilizer × Bobwhite control vs. GM	1
Fertilizer × Within Bobwhite control lines	3
Fertilizer × Within Bobwhite GM lines	3
Fertilizer × Frisal control vs. GM	1
Fertilizer × A9 vs. A13 (Within Frisal GM lines)	1
Residual	1115
Total	1199

3.3.2 Experiment 2: Pollen longevity of GM and non-GM wheat lines

For this experiment we did two different analyses. First, we analyzed pollen quality (% of stained pollen grains) without storage and checked there for the line differences in initial pollen quality. Then we analyzed separately the pollen quality after storage. Here we checked the effects of each treatment, interactions of the treatments, effects of lines and two-way interactions of the line contrasts and treatments (Fig. 18; Table 4).

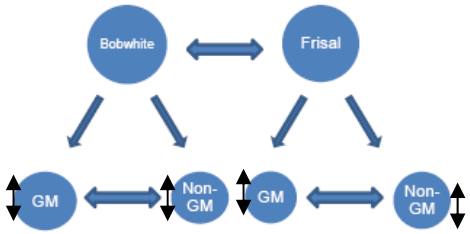


Fig.18. Hierarchical model of line comparisons of pollen experiment. Double-sided arrows show the comparisons with each other. Vertical arrows show the comparisons within GM and non-GM.

Table 4. Skeleton ANOVA table for the pollen experiment analysis after storage

Change	**d.f.**
Replicate	4
Treatments	
Temperature	1
Light	1
Temperature × Light interaction	1
Time	3
Temperature× Time	3
Light × Time	3
Line Contrasts:	
Frisal vs. Bobwhite	1
Bobwhite Control vs. GM	1
Within Bobwhite GM lines	1
Within Bobwhite control lines	1
Frisal Control vs. GM	1
Interactions Line × Treatments	
Temperature × Frisal vs.Bobwhite	1
Temperature × Bobwhite control vs. GM	1
Temperature × Within Bobwhite GM lines	1
Temperature × Within Bobwhite control	1
Temperature × Frisal control vs. GM	1
Light × Frisal vs. Bobwhite	1
Light × Bobwhite Control vs. GM	1
Light × Within Bobwhite GM lines	1

Light × Within Bobwhite control	1
Light × Frisal Control vs. GM'	1
Time × Frisal vs. Bobwhite	3
Time × Bobwhite Control vs. GM	3
Time × Within Bobwhite GM lines	3
Time × Within Bobwhite control	3
Time × Frisal Control vs. GM	3
Residual	433
Total	479

3.3.3. Experiment 3: Outcrossing ability of GM and non-GM wheat lines

For this experiment we did several kinds of analysis. The variable analyzed was number of seeds produced per spike. Bobwhite and Frisal varieties were analyzed separately because the plants were crossed within these groups and not between.

First, we analyzed main effects of mother and father plants to check if the number of seeds produced depended on which wheat line was used as a father or as a mother. Time of waiting before pollination was also added to the model for Bobwhite lines.

Then we analyzed separately the effects of different wheat lines when they were used as a pollen source or as mother plants (father effects, mother effects). As seen from the scheme (Fig. 19) and the ANOVA table (Table 5), effects of each treatment, interactions of the treatments, effects of the lines and interactions of the lines and treatments were fitted to the models. For Bobwhite lines, we did two different analysis: 1) we compared all *Pm3b* lines with all *S3b* control lines, then we compared two different *Pm3b* lines, and then two different *S3b* lines (within groups comparisons); 2) we compared genotypic pairs *Pm3b*#1+S3b#1 vs. *Pm3b*#2+S3b#2, then we made within pair comparisons *Pm3b*#1 vs. S3b#1 and *Pm3b*#2 vs. S3b#2.

We also analyzed the differences between cross-pollination and artificial selfing to check if more seeds were produced when the pollen came from the same genotype as stigma.

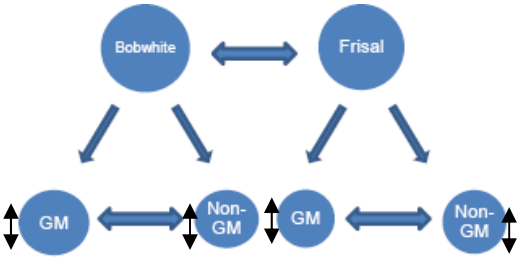


Fig.19. Hierarchical model of line comparisons of pollen experiment. Double-sided arrows show the comparisons. Vertical arrows show the comparisons within GM and non-GM.

Table 5. Skeleton ANOVA table for the analysis of outcrossing ability experiment

Testing mother and father plant main effects:	
Change	d.f.
Replicate	9
Time	1
Mother effect	3
Time × Mother effect	3
Father effect	3
Time × Father effect	3
Mother effect × Father effect	9
Time × Mother effect × Father effect	9
Residual	119
Total	159
Mother (or father) effects for Bobwhites with line contrasts, GM-Control comparisons:	
Change	d.f.
Replicate	9
Time	1
Pm3b#1 and *Pm3b*#2 vs. *S3b*#1 and *S3b*#2	1
Pm3b#1 vs *Pm3b*#2	1
S3b#1 vs. *S3b*#2	1
Time × *Pm3b*#1 and *Pm3b*#2 vs. *S3b*#1 and *S3b*#2	1
Time × *Pm3b*#1 vs *Pm3b*#2	1
Time × *S3b*#1 vs. *S3b*#2	1
Residual	143
Total	159
Mother (or father) effects for Bobwhites with line contrasts, pairwise comparisons:	
Replicate	9
Time	1
Pm3b#1 and *S3b*#1 vs. *Pm3b*#2 and *S3b*#2	1
Pm3b#1 vs. *S3b*#1	1
Pm3b#2 vs. *S3b*#2	1

Time × *Pm3b*#1 and *S3b*#1 vs. *Pm3b*#2 and *S3b*#2	1
Time × *Pm3b*#1 vs. *S3b*#1	1
Time × *Pm3b*#2 vs. *S3b*#2	1
Residual	143
Total	159
Selfing vs. outcrossing analysis:	
Change	d.f.
Replicate	9
Time	1
Selfcrossing	1
Replicate Time × Selfcrossing	9
Time × Selfcrossing	1
Residual	138
Total	159

4 Results

4.1 Results of experiment 1 (Seed longevity of GM and non-GM wheat lines)

4.1.1 Results of the initial germination test

Germination test, performed before the storage of the seeds in February 2009, showed no significant differences between GM and non-GM lines in seed germination ability (P=0.315; Fig.20). The seeds of Frisal and Bobwhite lines also did not differ in germination rate (P=0.407).

The three Swiss conventional wheat varieties (Casana, Fiorina and Toronit) differed in their seed germination ability (P=0.007). The variety Fiorina's germination rate was 5.5% lower than that of two other Swiss wheat varieties (91% of seeds germinated in Fiorina). Also there was no difference in the speed of germination between the studied wheat lines (how fast the seeds started to germinate; P=0.95).

Fertilization of the maternal plants (in NFP59 field trial 2008) did not affect germination ability of the seeds (P=0.068). The seeds from fertilized plants also did not start germination earlier then the seeds from plants that did not receive fertilizer in the field (P=0.665).

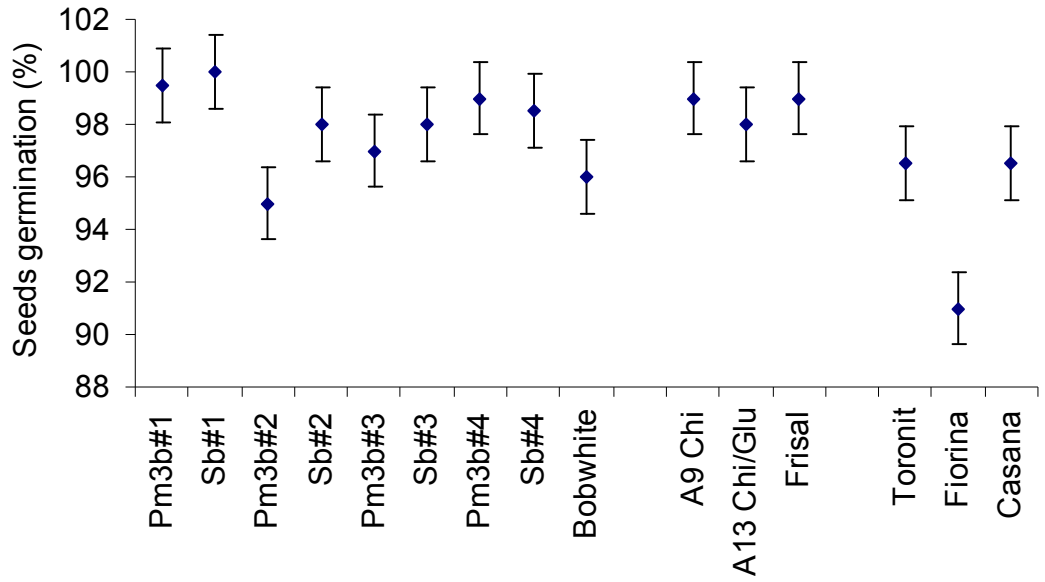


Fig.20. The results of the initial germination test performed before the storage.

4.1.2. Results of the seed germination during storage

Most of the seeds germinated during storage. The seeds which didn't germinate during storage also didn't germinate later in the germination test. These seeds were counted as "not alive".

Time of storage had a significant effect on the percentage of seeds germinated during storage ($P<0.001$). More seeds (90.96%) germinated during storage for a longer period of time (six months) than when they were stored for a shorter period (three months, 82.98% germinated).

Oxygen availability significantly affected seed germination ($P<0.001$). Although the seeds germinated in both aerobic and anaerobic conditions, higher rate of germination was found in the presence of oxygen (97.2%) than in anaerobic soil (76.73%). Observed seed germination in anaerobic conditions might be explained by the presence of some oxygen between soil particles in Petri dishes before it was fully replaced by nitrogendioxide. Apparently, this amount of oxygen was enough for seeds to start germination. There was a difference, however, in the length of roots between aerobic and anaerobic treatments (Fig. 21). The roots grew for a longer period of time in aerobic treatment and were - according to our visual observations - up to five times longer in aerobic conditions than in anaerobic treatment.

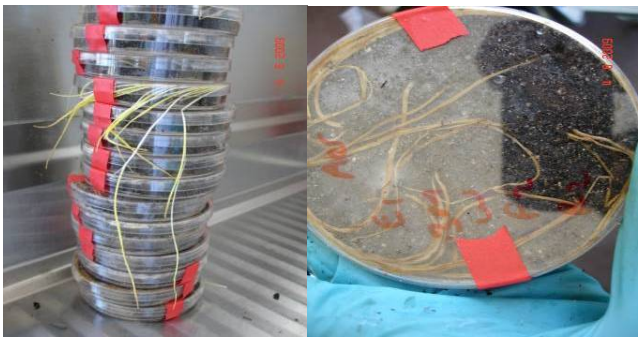


Fig.21. The seeds germinated in aerobic conditions.

Soil humidity also played an important role in seeds germination ability (P<0.001). More seeds germinated in dry soil (17.6 % humidity) than in wet soil (77.5% humidity) conditions. This might be related to the oxygen shortage in wet soil. 17.6% of soil humidity in “dry soil” treatment was sufficient for seeds to germinate.

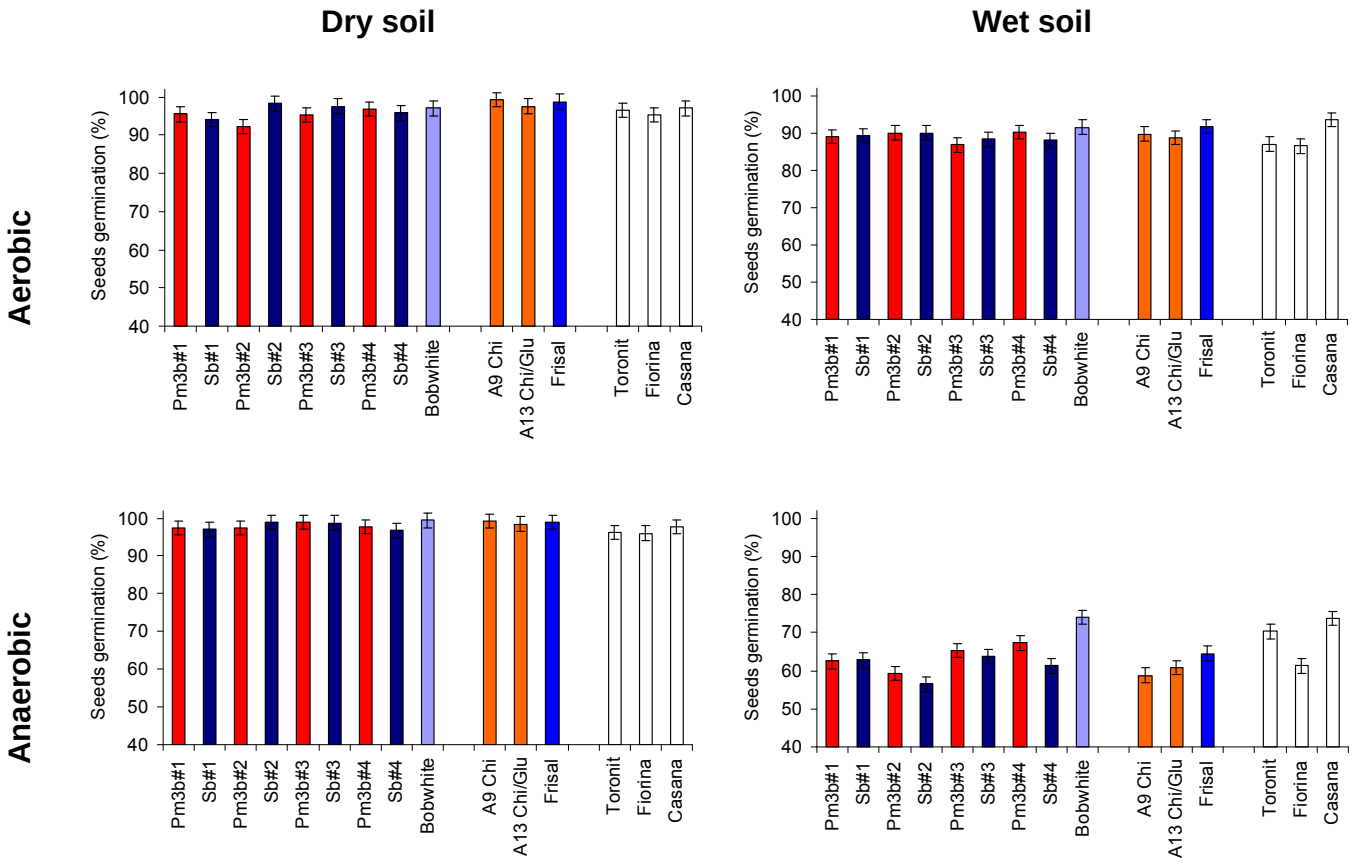


Fig.22. Seeds germination under different storage conditions.

There was a significant interaction between humidity and oxygen availability conditions (P<0.001).In aerobic conditions water availability played a positive role in seed germination (more seeds germinated in wet soil then in dry soil), whereas, in anaerobic conditions water played a negative role and caused a decrease in germination rate (Fig.23)

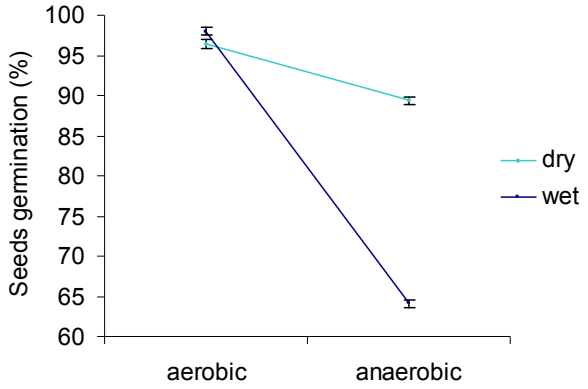


Fig.23. Seeds germination at two soil moisture conditions in the presence and absence of oxygen.

Under wet soil conditions, there were differences in germination rates between Swiss conventional varieties. Fiorina had significantly lower (P<0.001) germination rate under anaerobic wet conditions compared to other Swiss varieties. Under wet aerobic conditions, Toronit and Fiorina showed lower germination ability than variety Casana (P<0.001).

Generally, no differences between GM and non-GM were found (P=0.643 for the difference between *Pm3b* and *Sb* lines, P=0.097 for Frisal vs. A9 and A13). Only *Pm3b*#2 line showed significantly lower germination rate (92.25%) compared to control *S3b*#2 line (98.25%) when stored under aerobic dry conditions (P<0.001).

Under wet anaerobic conditions of storage, the seeds of the mother variety Bobwhite germinated better (P<0.001) than those of sister lines (*S3b*#1-4). Under other storage conditions this difference was not found.

Summarizing the results of this experiment, we found no indication that the seeds of studied GM lines can persist longer in the soil then their corresponding controls at given humidity, oxygen and temperature of storage. Most of the seeds germinated quickly even at oxygen and water shortage conditions. None of the seeds that did not germinate survived storage in the soil. One of the lines (*Pm3b*#2) showed lower seed germination rate than its corresponding control when the seeds were stored under aerobic dry conditions. Water availability in the soil was advantageous for seed germination in aerobic conditions but a disadvantage in the shortage of oxygen.

4.2 Results of Experiment 2 (Pollen longevity of GM and non-GM wheat lines)

4.2.1 Initial pollen viability before storage

Viability of pollen of six wheat lines was checked immediately after pollen was collected from the plants before starting the experiment with storage conditions. Initial viability of pollen of GM and non-GM lines were equal (P=0.291 for *Pm3b*#1-2 vs. *S3b*#1-2 lines, P=0.71 for Frisal vs. A9). The percentage of viable pollen was lower for the lines of Frisal variety than for Bobwhite family (P<0.001). This might be due to the genetic differences between two mother varieties and the earlier start of flowering of Swiss variety Frisal compared to Mexican wheat variety Bobwhite (Fig. 24). Although we tried to collect pollen from the spikes of the same flowering stage, the age of pollen of Frisal and Bobwhite lines might be slightly different.

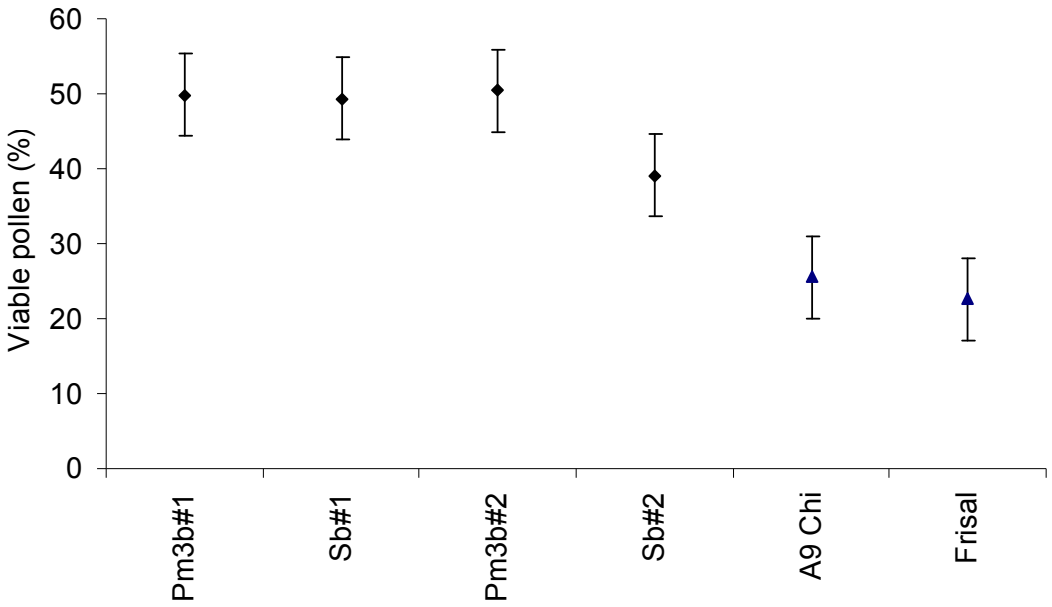


Fig.24. Pollen viability of transgenic and control wheat lines before the storage.

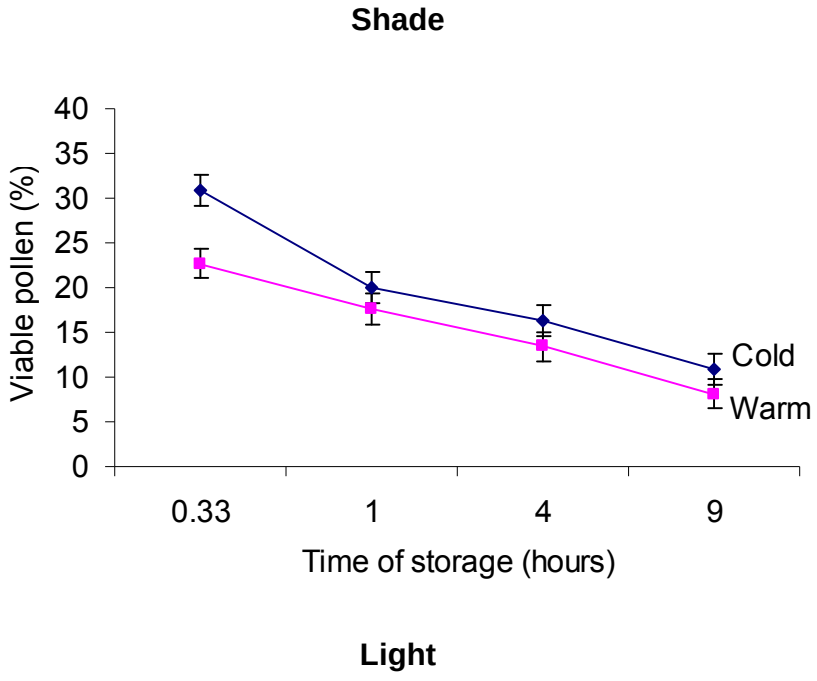
4.2.2 Pollen viability after storage

The amount of alive pollen declined with a time of storage (P<0.001). However, even after nine hours of storage we found up to 12% of stained pollen. This indicates that, under

certain environmental conditions, pollen of wheat might keep viability longer than it is usually reported in the literature.

Temperature significantly affected viability of stored pollen (P<0.001). Pollen stored under cold conditions (10°C) survived longer then the pollen kept at 25°C.

Generally, light conditions did not affect pollen viability (P=0.362 for the main effect). However, there was a significant interaction Light x Temperature (P=0.032): in the shade pollen viability was similar at cold and warm conditions, whereas in the light presence cold conditions prolonged pollen viability (Fig. 25).



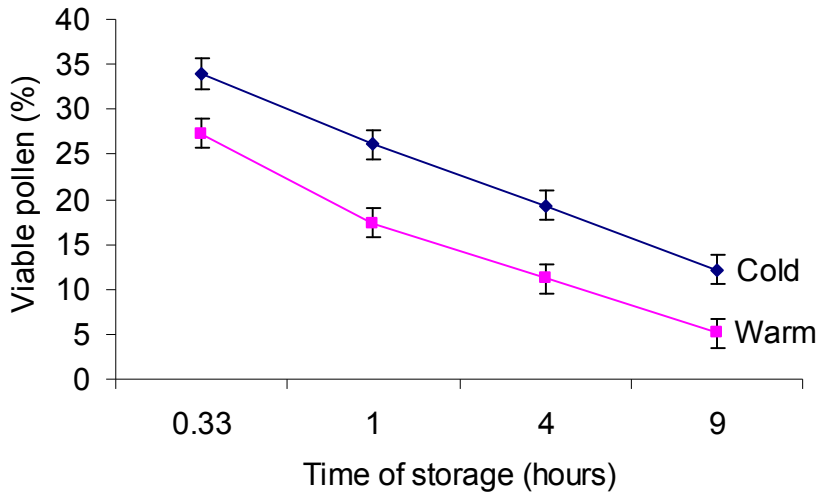


Fig.25. Wheat pollen viability after storage in different light and temperature conditions.

Wheat lines of Frisal family, generally, showed milder decline in pollen quality over time than lines of Bobwhite family ($P<0.001$). However, this difference was dependent on the temperature and time of storage ($P<0.001$ for the interactions Frisal.Bobwhite x Temperature and Frisal.Bobwhite x Time). The difference Bobwhite vs. Frisal became smaller at warm conditions since the quality of Bobwhite pollen declined stronger than that of Frisal family. Although, initially the quality of pollen of Frisal lines was lower than that of Bobwhites, with the increase of time of storage the difference between Frisal and Bobwhite families decreased and after nine hours of storage disappeared (Fig. 26).

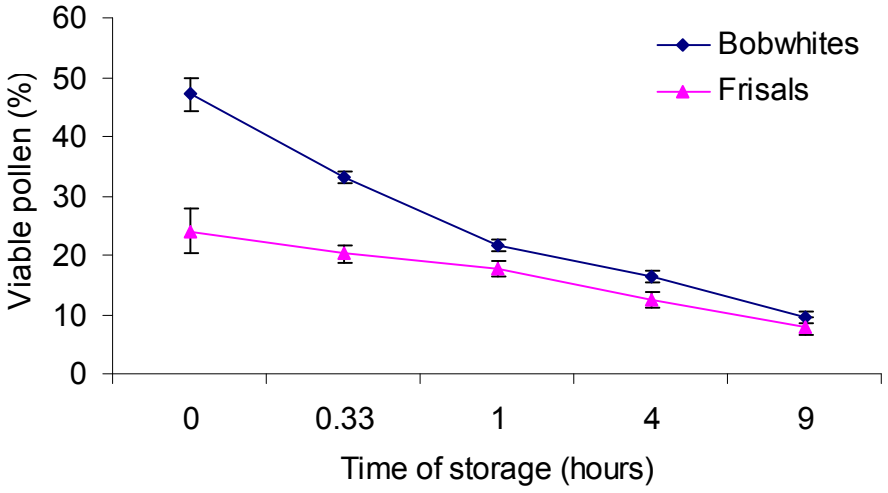


Fig.26. Pollen viability of the lines of Bobwhite and Frisal families after storage for a certain period of time.

There was no difference between GM *Pm3b* lines and non-GM S3b lines (P=0.351). A9 *Chi* line differed from its mother variety Frisal (P=0.001), showing slightly higher pollen viability at some time points of storage (Fig. 27).

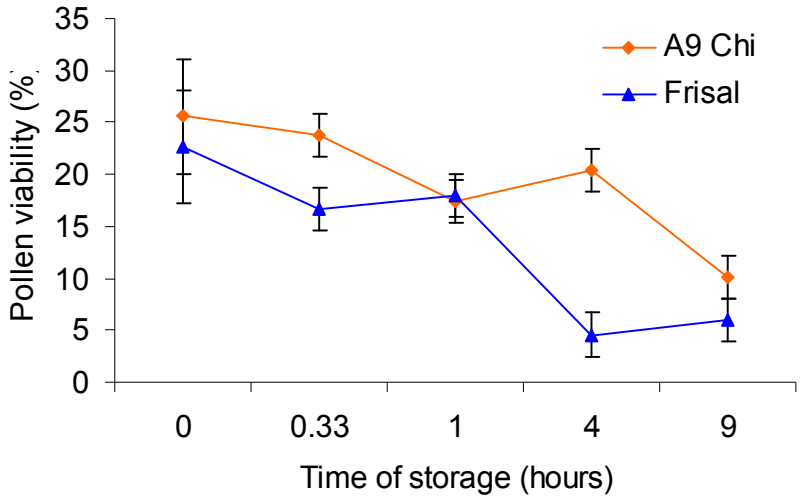


Fig.27. Pollen viability of GM A9 *Chi* line and non-GM wheat variety Frisal after storage for a certain period of time.

All other lines did not differ in their pollen viability. Finally, after nine hours of storage there was no difference in pollen viability between all the studied lines (Fig. 28).

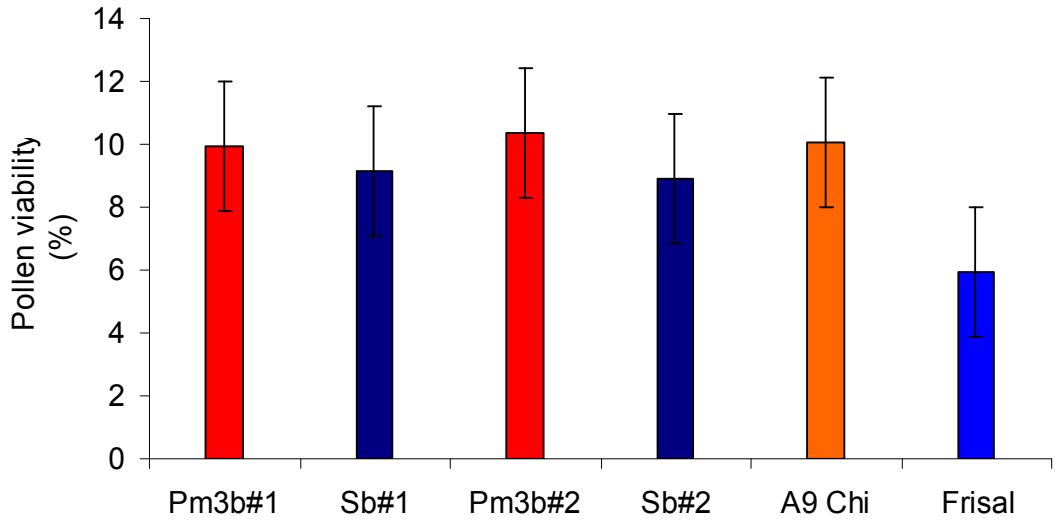


Fig.28. Pollen viability of GM and non-GM wheat lines after 9 hours of storage.

Summarizing the results of pollen experiment, there was no difference in pollen longevity or initial pollen quality between GM and non-GM lines.

Under studied conditions, pollen of the wheat lines kept viability for nine hours.

The lines of Frisal family had initially lower pollen quality than those of Bobwhite family.

Pollen survived longer under cold conditions than under higher temperature.

4.3 Results of Experiment 3 (Outcrossing ability of GM and non-GM wheat lines)

Hybridization of GM and non-GM wheat lines was successful. All the hybrid combinations planned, both direct and reciprocal, were obtained (Tables 6, 7).

Table 6. Average number of hybrid seeds obtained per crossing combination of the lines of Bobwhite family

♀ \ ♂	*Pm3b*#1	*S3b*#1	*Pm3b*#2	*S3b*#2
Pm3b#1	17.8	7.9	17.4	22.8
S3b#1	17	13	11.5	21
Pm3b#2	14.3	13.5	15.6	15.6
S3b#2	16.4	15.9	10.6	21.1

s.e. = 2.66, n = 10.

Table 7. Average number of hybrid seeds obtained per crossing combination of the lines of Frisal family

♀ \ ♂	A9 *Chi*	Frisal
A9 *Chi*	6.444	7
Frisal	3.3	4.778

s.e. = 2.66, n = 10.

In the analysis of “father” effects there was a significant difference found between *Pm3b* and *S3b* lines (P=0.036). However, this effect was fully explained by the difference between *Pm3b*#2 and *S3b*#2 lines: significantly lower number of seeds was produced in the crossings where the pollen was taken from *Pm3b*#2 line compared to the crossings with *S3b*#2 line as a father (P=0.009). Other GM lines did not differ from their corresponding controls (Fig. 29).

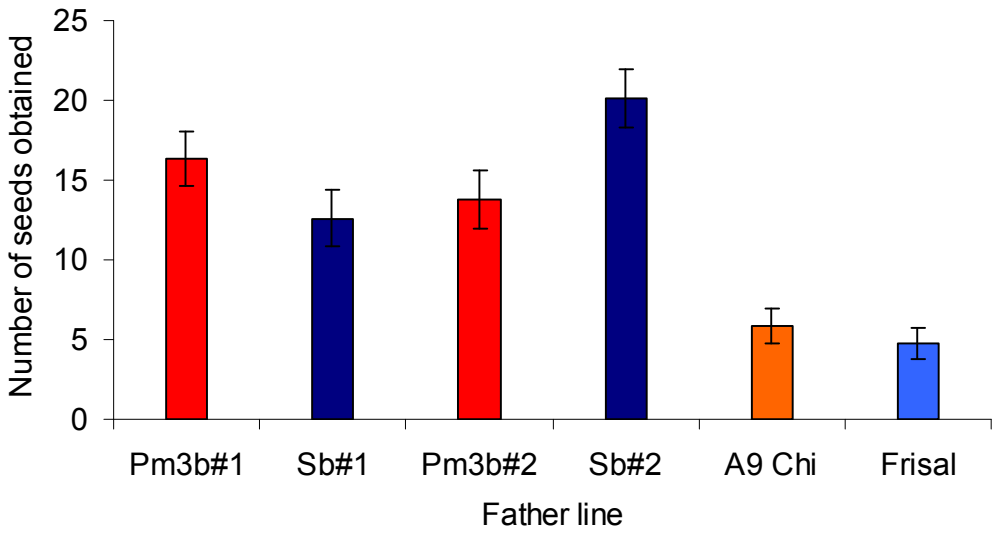


Fig.29. Effect of the wheat line used as a father on the efficiency of cross-pollination.

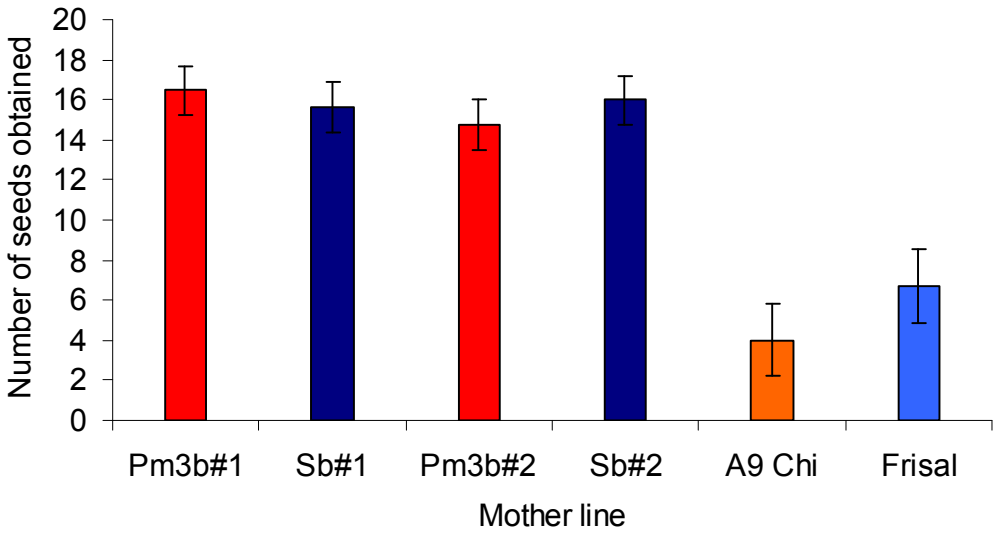


Fig.30. Effect of the wheat line used as a mother on the efficiency of cross-pollination.

No differences were found between GM lines and non-GM lines in the efficiency of cross-pollination when they were used as mother plants for crossings.

On average, less seeds were obtained in crosses of the lines of Frisal family than those of Bobwhite family ($P<0.001$). This might be due to natural differences between wheat

varieties in their flower morphology or pollen quality that would affect the efficiency of artificial pollination.

The first crossings were made immediately after castration of the mother spikes and were not very successful. Then we changed the technique and performed pollination in three days after the anthers were removed from the mother plant. This allowed stigmas of the mother flowers to develop properly and significantly increased the efficiency of hybridization ($P<0.001$; Fig. 31).

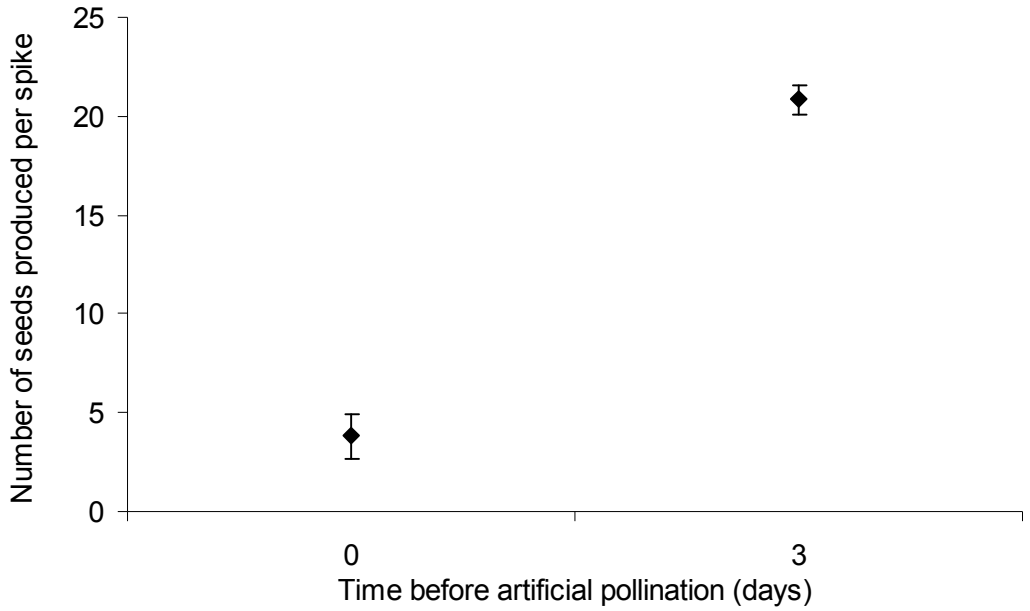


Fig.31. Effect of the time period between the castration of the spike and artificial cross-pollination on the efficiency of hybridization.

No differences in the number of seeds produced were found between artificially self-pollinated and artificially cross-pollinated plants ($P=0.061$). Reciprocal crossing combinations also did not differ significantly in the number of seeds obtained per spike ($P=0.192$).

Summarizing, it was found that less seeds were produced by the spikes pollinated with pollen from *Pm3b*#2 GM line than those pollinated with pollen from non-GM line *S3b*#2 (father effect). Since in the experiment with pollen we found no difference in pollen quality between *Pm3b*#2 and *S3b*#2 lines, one of possible explanations of lower

hybridization rate might be smaller amount of pollen per spike in *Pm3b*#2 line. This assumption will probably be tested in the field later. No differences between GM and control lines were observed when they were used as mother plants for crossings. Crosses were more efficient if three days were allowed between spike castration and pollination.

5 Discussion

In the literature, the studies on germination percentage of wheat seeds indicated a decrease of seed viability with the increase of age. Storage conditions, particularly temperature and moisture have been indicated as the main factors influencing seed longevity (Akhter *et al*. 1992). We found that most of the wheat seeds germinated quickly even at oxygen and water shortage conditions. None of the seeds that did not germinate survived storage in the soil. We found no indication that the seeds of studied GM lines can persist longer in the soil then their corresponding controls at given humidity, oxygen and temperature of storage. One of the lines *Pm3b*#2 showed lower seed germination rate than its corresponding control under aerobic dry conditions of storage. Water availability in the soil was an advantage for seed germination in aerobic conditions but a disadvantage in the shortage of oxygen.

From the literature (De Vries 1971; Luna *et al*. 2001; Loureiro *et al*. 2007), it is known that the ability of pollen to affect seed set decreases with the time of storage and the temperature increase. It has been reported (Loureiro *et al.* 2007) that no viable pollen can be left after 3 h.

We found that under studied conditions, pollen of the wheat lines kept viability for 9 hours.

There was no difference in pollen longevity or initial pollen quality between GM and non-GM lines, as referred in the studies of Dale, Clarke & Fontes (2002) and Kang *et al.* (2009).

The lines of Frisal family were characterized by initially lower pollen quality than those of Bobwhite family. In our experiment, pollen survived longer under cold conditions than under higher temperature. This does not correspond to the results obtained by Luna *et al.* (2001) and Wang *et al.* (2004).

We found that less seeds were produced by the spikes pollinated with pollen from *Pm3b*#2 GM line than from those pollinated with pollen from non-GM line *S3b*#2 (father effect). Since in the experiment with pollen we found no difference in pollen quality between *Pm3b*#2 and *S3b*#2 lines. One of possible explanations of lower hybridization rate might be smaller amount of pollen per spike in *Pm3b*#2 line. This assumption will probably be tested in future experiments in the field. No differences between GM and non-GM lines were observed when they were used as mother plants for crossings. Crosses were more efficient if 3 days were allowed between spike castration and pollination. This supports recommendations of Meister & Tjumjakoff about artificial hybridization procedure in wheat.

6 Conclusions

The seeds of the GM wheat lines do not persist longer in the soil than the seeds of corresponding control lines at the humidity, oxygen and temperature conditions of storage used in the experiment.

Being stored in soil, most of the seeds (90-100%) of studied 16 wheat lines germinated quickly. The seeds that did not germinate were also not viable after storage.

Water availability in the soil was advantageous for seed germination in aerobic conditions but a disadvantage in the shortage of oxygen.

Under studied environmental conditions, pollen of wheat kept viability for nine hours (up to 12% of viable pollen after nine hours of storage).

Transgenic wheat lines did not differ from corresponding control in pollen quality and longevity.

The lines of Frisal family characterized by initially lower pollen quality (% of viable pollen) than those of Bobwhite family, probably, due to the variety differences in pollen viability or in flowering time.

Pollen survived longer under cold conditions (10^0C) than under warm conditions (25^0C).

The efficiency of hybridization was lower when *Pm3b*#2 GM line was used as a pollen source. Since the quality of pollen of this line did not differ from others we can assume that the quantity of pollen produced per spike might be smaller.

7 Acknowledgments

I would like to thank Olena Kalinina for her supervising, patience and help in experimental design and analysis throughout my experiment. Also special thanks to Prof. Bernhard Schmid for supervising the project. I would also like to thank Rene Husi for his help in the laboratory and all the people at the former Institute of Environmental Sciences. I am especially grateful to my husband for believing and supporting me to complete this work.

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